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# A New Procedure for DNA Isolation from Saliva Samples and Comparative Analysis of Quality Indicators

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## Abstract

**Background:** Large population-based studies involving thousands of participants are needed for research on genetic diseases and epidemiologic studies. Saliva samples are a non-invasive and efficient DNA source for mass collection. The establishment of new optimized DNA isolation procedures from saliva and the determination of the most effective quality indicator are essential for this purpose.

**Methods:** DNA was extracted from 112 saliva samples utilizing a novel method. Samples were pre-treated with Protease for 1 h at 56°C, and reagents from a kit for blood samples were used in the Chemagic MSM-I automated instrument with a specifically designed saliva protocol for the Chemagic software. DNA quality was estimated by spectrophotometry, fluorometry, qPCR, SPUD assay and the Agilent 2200 Tape Station.

**Results:** An average DNA yield of 52.58 ± 33.77 µg was obtained with no significant differences between males and females. $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios of 1.84 ± 0.123 and 1.56 ± 0.297 were obtained respectively. A DIN value of 6.83 ± 0.90 was observed with a satisfactory functionality calculated by qPCR analysis. On the other hand, significant differences were observed between spectrophotometry, fluorometry and qPCR quantification methods in spite of the low amount of contaminants detected.

**Conclusion:** Collecting as many samples as possible is necessary to establish DNA cohorts that represent the whole population. The non-invasive procedure described in this work guarantees a large amount of DNA from saliva samples valid for any downstream molecular applications, with an important reduction in costs. Additionally, an innovative comparison between the DIN values and conventional DNA quality indicators is shown.

**Keywords:** Epidemiologic studies; Saliva DNA; DNA quality control; Personalized medicine; DNA banking

## Introduction

The current demand for personalized medicine, the importance of genetic information in drug development and clinical trials and the great magnitude of epidemiological studies have made the routine collection of DNA samples [1,2] necessary. Large population-based studies involving thousands of participants are needed for the study of genetic diseases and epidemiologic studies [3]. Mass collection of blood samples for DNA extraction is sometimes impossible because it requires venepuncture by trained staff, which makes sample collection expensive. Therefore, less invasive and cost-efficient procedures for obtaining DNA are needed [3]. Because of the previous reasons, saliva samples emerge as a promising alternative [4]. In spite of it being known that the co-extraction and contamination of human DNA with a significant proportion of bacterial and viral DNA can occur [5]. DNA banks constitute an important repository of samples, which are collected, processed, and stored in accordance with rigorous quality criteria [6]. A sufficient amount of high-quality DNA must be available for downstream applications [7]. Optimization and validation of new processing methods for DNA purification from different samples is necessary for biobanks as part of their quality assurance systems [6]. On the other hand, it is essential to establish a standardized and cost-effective workflow [8]. Specific and high-throughput sample preservation products and nucleic acid extraction methods from saliva have appeared in recent years, with a low processing time and user variability [9]. Most of the commercial protocols have been optimized and coupled to commercial devices such as Oragene (DNA Genotek), SalivaBio (Salimetrics, Carlsbad, USA), and Norgen Saliva DNA preservative (Ontario, Canada) for saliva collection, stabilization and storage until DNA extraction. However, in spite of their advantages, the use of these commercial products involves a high cost per sample. The usability of DNA for analytical techniques is determined through DNA quality indicators (purity and integrity). To establish the concentration and purity of DNA samples, the most widespread and routine method is spectrophotometry [10]. The absorbance ratio between 260 nm and 280 nm is used to estimate DNA purity with a value of 1.8-2.2 considered as "pure" DNA [11]. The 260 nm/230 nm absorbance ratio is used as a secondary measure of DNA purity [12]. Expected 260/230 values for "pure" DNA are commonly within the range between 2.0 and 2.2, although it is considered a questionable DNA quality indicator because of its instability when a saline elution buffer is used to dissolve the DNA [13]. Additional and more selective methods based on fluorometry such as PicoGreen, Qubit or qPCR, are used for DNA quantification [5]. The presence of inhibitors from nucleic acid extraction reagents or co-purified components in DNA samples is an important issue when they are used in enzymatic

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or amplification downstream applications [14]. Different methods are used to check PCR inhibition such as "SPUD" assay [14]. Other authors simply test the absence of inhibitor by genotyping specific gene fragments [3,15,16]. DNA integrity has traditionally been checked by electrophoresis in agarose gel. However, the importance of genomic DNA quality for some downstream applications [8], has led to the development of systems that assess DNA integrity in an objective and standardized way. In this sense, 2200 Tape Station instrument (Agilent Technologies) provides a numerical determination of the DNA integrity called DNA Integrity Number (DIN). The DIN algorithm was developed to remove user-dependent interpretation of DNA quality and to provide a standardized assessment [17]. In this work we show a novel adapted method for saliva DNA extraction without the use of specific and expensive devices for sample collection. DNA concentration and purity, as well as integrity and functionality, have been analysed by different methods and instruments. A comparison between the results obtained is presented and discussed in order to propose the most effective quality indicator.

## Materials and Methods

### Human biological samples

Samples and information related to age and gender were collected from 112 healthy donors aged between 18 and 45 years old. Samples were codified, except for 4 donors that were anonymized (leaving 23 males and 85 females). Handling of human biological samples was carried out according to the national legal framework (Spanish Law on Biomedical Research (July 2007)) following informed consent from the donors. Local scientific and ethics committees approved the procedures performed in this work (Project number PI-0414-2014). Participants were asked to spit saliva into a sterile 15 ml conical tube up to the 2 ml mark without worrying about bubbles. Participants were warned not to drink, eat, smoke, brush their teeth or chew gum during the hour prior to sample collection. The participants rinsed their mouths with water prior to collection and waited 10 minutes before commencing the collection. Samples were immediately put into storage at -20°C until processing [18].

### DNA extraction

DNA from the 2 ml saliva samples was extracted by using the Chemagic MSM-I Magnetic Separation Module (PerkinElmer Inc., Massachusetts, EEUU) based on magnetic beads. Specifically, magnetic beads are conjugated with polymers to capture the DNA liberated after cell lysis and protein degradation [19]. By means of an electromagnetic field, the beads are attracted to magnetized metal rods. DNA is washed with different washing buffers to be finally eluted in elution buffer, which breaks down the interaction between the beads and the DNA. Reagents from a kit for blood samples (Chemagic DNA Blood Kit special 3 ml, Cat n°CMG 763-1; PerkinElmer Inc.) were used to isolate DNA from saliva, but using a specifically designed saliva protocol for the Chemagic software from the manufacturer, "chemagic DNA saliva4k H12 prefilling VD110715.che". Before introducing samples into the automated machine, a previous manual lysis step was performed by adding 2 ml of lysis buffer and 45 µl of Protease supplied in the kit to each sample, which were incubated for 1 h at 56°C in a water bath.

### DNA quantification and purity estimation

Different methods and instruments were used for quantification and purity assessment of DNA samples. All the samples were analysed by spectrophotometry with the Infinite F200 (Tecan Trading AG, Männedorf, Switzerland). On the other hand, 12 samples were randomly selected for a comparative analysis by using the spectrophotometer Nanodrop One (Thermo Fisher Scientific Inc., Massachusetts, EEUU), the fluorometer Qubit 1.0 (Thermo Fisher Sientific Inc., Massachusetts, EEUU) and the Genomic DNA Quantification Assay kit based on quantitative PCR (Primerdesign, Southampton, England). Additionally, the presence of contaminant RNA in these 12 DNA samples was determined in the Qubit 1.0, and the presence of PCR inhibitors with the SPUD assay.

### Infinite F200 spectrophotometer

Yield and purity were spectrophotometrically determined for all the samples by using the Infinite F200; 2 µl of each sample was used in duplicate and the average value was determined. Concentration was calculated considering the $A_{260}$ absorbance value and yield taking into account the elution volume. Purity was estimated with the $A_{260}/A_{280}$ nm ratio and $A_{260}/A_{230}$ nm ratio as a secondary value.

### Nanodrop™ One C spectrophotometer

Twelve samples were spectrophotometrically measured with the Nanodrop One; 2 µl of each sample was used in duplicate and the average value was determined. $A_{260}$ absorbance value was used to calculate the concentration, and yield was estimated using the elution volume. $A_{260}/A_{280}$ and $A_{260}/A_{230}$ purity ratios were also obtained. The absorption spectrum between 220 nm and 350 nm was also obtained to analyze the presence of DNA contaminants.

### Qubit 1.0 fluorometer

Twelve samples were measured by fluorometry using the Qubit 1.0 device (Thermo Fisher Scientific Inc., Massachusetts, EEUU) and Qubit dsDNA BR Assay kit (Thermo Fisher Sientific Inc., Cat. No. Q32850), for DNA sample concentrations between 100 pg/µl and 1 µg/µl, or Qubit RNA HS Assay Kit (Thermo Fisher Scientific Inc.; Cat. No. Q32852), for RNA sample concentrations between 250 pg/µl and 250 ng/µl. 5 µl of each sample was used, following the manufacturer's instructions. Three readings were taken per sample and the average value was calculated. DNA yield was estimated by means of the elution volume.

### qPCR quantification assay

Genomic DNA Quantification Assay (Primerdesign Ltd.; Cat. No. gDNA-hu-q-DD) and PrecisionPlus 2 × qPCR MasterMix (Primedesing Ltd.; Cat. No. Mini-PrecisionPLUS) were used in a LightCycler 96 thermocycler (Hoffmann-La Roche, Ltd., Basilea, Switzerland) following the manufacturer's instructions to estimate the DNA concentration. The kit provides specific primers for the amplification of a 156 bp single copy region of non-transcribed DNA. A standard curve was generated using the positive control provided by the kit and a negative control reaction was included to discard the presence of contamination, following the manufacturer's instructions. 25 ng of DNA was used per reaction, considering the fluorometric concentration, in a 15 µl final volume.

### SPUD assay

A quantitative PCR *assay* for the detection of PCR inhibitors in nucleic acid preparations (SPUD assay) [14] was performed in the 12 DNA samples selected. An artificially synthesized 101 bp template based on the *Solanum tuberosum phyB* gene fragment, a specific pair of primers (Sigma-Aldrich Co. LLC., Missouri, EEUU) for the amplification of this fragment and 50 ng of DNA, considering the spectrophotometric measure, were added to the 25 µl reaction volume.

KiCqStart SYBR green qPCR Ready Mix (Sigma-Aldrich Co. LLC.; Cat. No. KCQS00) and LightCycler 96 (Hoffmann-La Roche Ltd.) instrument were used. A negative control was included with Ultrapure DNAse/Rnase-free distilled water (Thermo Fisher Scientific Inc.; Cat. No. 10977-035) instead of DNA.

### DNA integrity and functionality 2200 TapeStation Software

Microfluidic DNA electrophoresis in the 2200 TapeStation (Agilent Technologies Ind., California, EEUU) was performed with the Genomic DNA ScreenTape (Agilent Technologies Inc.; Cat No. 5067-5365) and Genomic DNA Reagents (Agilent Technologies Ind.; Cat No. 5067-5366) for the 112 DNA samples. For integrity estimation, the TapeStation software calculated the DNA Integrity Number (DIN), with values between 1 and 10, considering 1 as highly degraded DNA and 10 as DNA without degradation. Concentration values were also obtained.

### PCR amplification

Thirty randomly selected DNA samples were amplified by real time PCR in a Roche Light Cycler 96 Instrument for the GAPDH, AF4 and ZFX genes. PCR products of 87, 400 and 1137bp were obtained respectively. 5 ng of DNA from each sample was employed in 20 µl amplification reactions containing 10 µl of FastStart Essential DNA Green Master (Hoffmann-La Roche, Ltd., Basilea, Switzerland, cat n° 06402712001) and 0.4 mM GAPDH, 0.4 mM AF4 or 0.8 mM ZFX primers as described previously [6]. A PCR program with an initial denaturation step of 10' 95°C, and 40 cycles at 95°C for 20'', 60°C for 20'' and 72°C for 20'' was used. A melting curve was added to check the absence of unspecific amplification.

### Statistical analysis

The SPSS Statistic (IBM, NY, EEUU) program was used for statistical analysis. Descriptive statistics module was used for the analysis of the mean and standard deviation. Histograms were constructed, in order to observe the shape of the distributions involved in the study. Given the size of the populations analyzed, the normality of the variables was studied with the Kolmogorof-Smirnov test for women, and with the Shapiro-Wild test in the case of men. It was concluded that the variables did not present a normal distribution, so the non-parametric Mann-Whitney-Wilcoxon test was applied to study the existence of significant differences for the variables between males and females.

## Results

### Yields and quality after DNA extraction from saliva

When DNA was isolated from the 112 saliva samples, a high variability of DNA yields was obtained with an average of 52.58 ± 33.77 µg (Figure 1a), although it was not affected by gender as no significant differences were found between males (51.22 ± 37.42) and females (54.78 ± 32.58). Consistently, DNA concentration was 133.6 ± 83.74 ng/µl and high enough for downstream applications. DNA purity was estimated by spectrophotometry with the Infinite F200. An optimum $A_{260}/A_{280}$ ratio of 1.84 ± 0.123 was observed (Figure 1b). In addition, a high $A_{260}/A_{230}$ ratio of 1.56 ± 0.297 was obtained (Figure 1c). A DIN value of 6.83 ± 0.90 was observed when we examined the DNA integrity by 2200 TapeStation (Figure 1d). No statistically significant differences were obtained between males, 6.97 ± 0.93, and females 6.8 ± 0.91 (p >

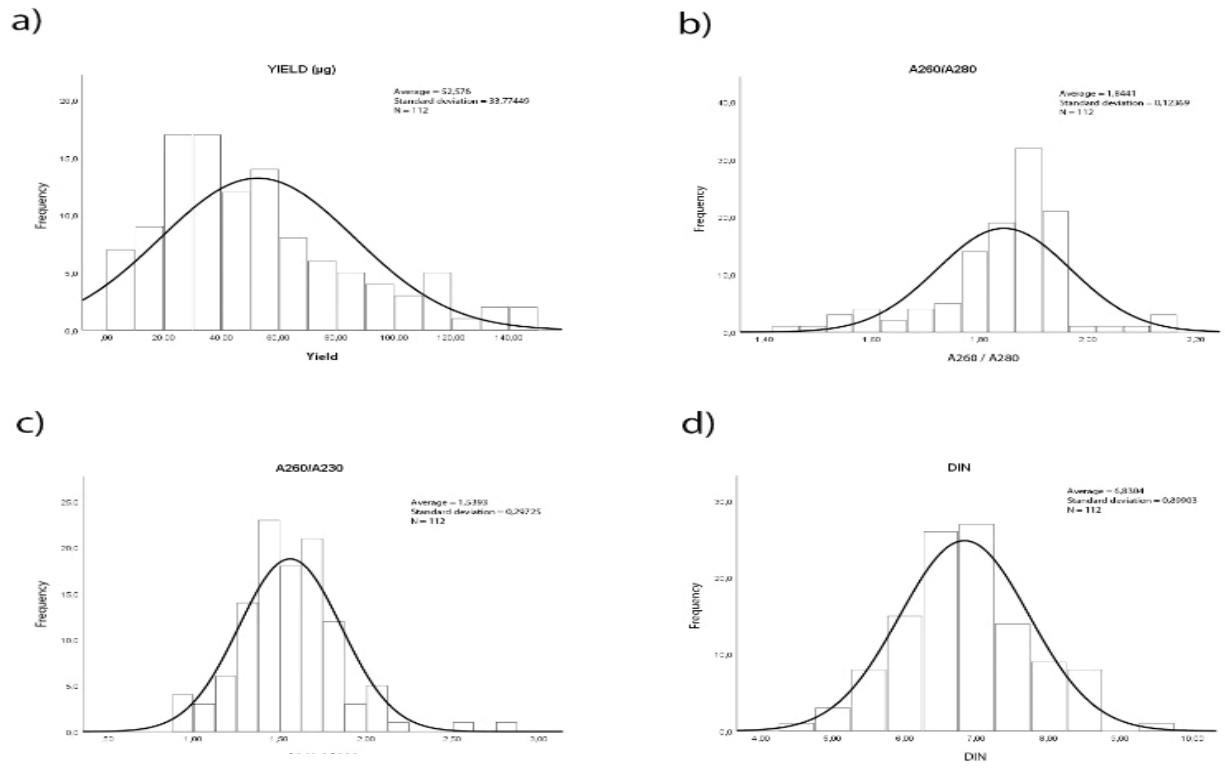


**Figure 1:** Histograms shown yields (µg) calculated from spectrophotometric measures with the Infinite F200 (a) $A_{260}/A_{280}$ (b), $A_{260}/A_{230}$ (c) and DIN values (d) purity ratios obtained for the 112 DNA samples isolated. Average, standard deviation and normal curve are indicated in the plots.

0,05). Representative samples for a range of DIN values can be seen in Figure 2. The purified DNA functionality was checked in 30 randomly selected samples by real time PCR amplification of three different sizes of PCR fragments GAPDH, AF4 and ZFX (87, 400 and 1137 bp respectively). Amplification of all three fragments was obtained for all the samples, except for one sample which failed to amplify ZFX gene. Average Cq values of 19.2, 20.5 and 31.5 were obtained for GAPDH, AF4 and ZFX genes respectively (Figure 3).

## Comparison of DNA quality methods

In order to check the reliability of previous spectrophotometric measures, DNA purity and concentration were determined by different methods for a group of 12 randomly selected samples. Results are shown in Table 1 and Figure 4. Concentration and yield calculated from $A_{260}$ absorbance, and $A_{260}/A_{280}$ and $A_{260}/A_{230}$ purity ratios, were similar for both the Nanodrop OneC and Infinite F200 spectrophotometers, in spite of the severe and concentration dependent $A_{260}/A_{230}$ nm fluctuation (Figure 4). Absorption spectra obtained with the Nanodrop™ OneC instrument showed a single well defined absorption peak at 260 nm, with a higher signal for the most concentrated samples [1,3,7,11,12]. Regarding concentration and yield obtained by using the Qubit 1.0 fluorometer and qPCR quantification assay, these values were quite similar, while higher values were obtained with the 2200 TapeStation (Table 1). However, all of them were considerably lower in comparison with the spectrophotometric results on the other hand, RNA presence was quantified by fluorometry (Qubit 1.0), with very low contamination detected, ranging from 1 to 15.1 ng/µl (4.46 ± 4.03 ng/µl) (Figure 4). When the detection of inhibitors was checked for the DNA samples by SPUD assay, less than one Cq of difference was obtained in relation to the negative control, with a slight displacement of the amplification curve, except for 2 samples (S11 and S12) which showed some minimal inhibition (Figure 5).

## Discussion

Traditionally blood samples have been used as a source of nucleic acids for research. However, the invasive nature of this type of sample has led to the search for alternative fluids such as saliva for guaranteeing a high participation of subjects in large-scale studies [20]. Saliva is a important source of nucleic acids for molecular diagnostic techniques

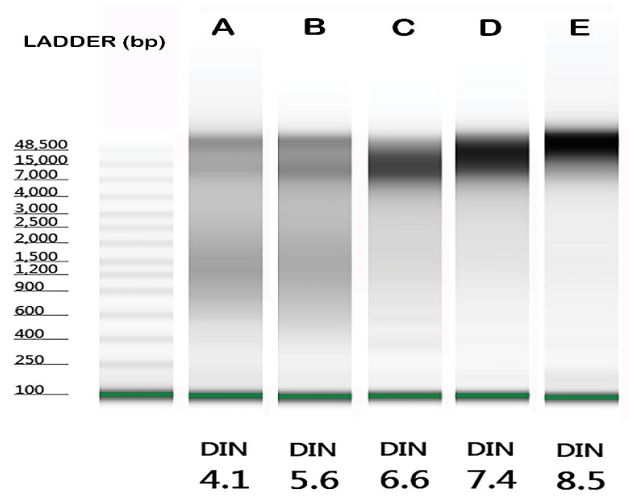


**Figure 2:** Representative smears for DNA samples ranged from 4.1 to 8.5 DIN values.

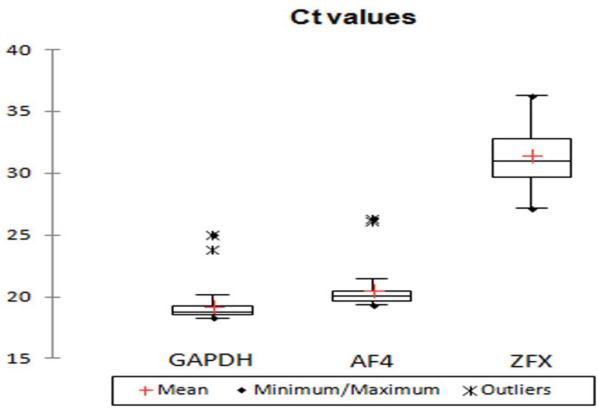


**Figure 3:** Diagram of Cq values for GAPDH, AF4 and ZFX genes. Box-plots of the data have been represented; Mean is indicated with "+" sign. The box with horizontal black line shows the first and third quartiles and the median. The whiskers show 1,5 times the interquartile range and the points show outliers. Outliers are indicated.

| Variables | Concentration (Ng/µl) | Yield (µg) |
|---|---|---|
| Infinite F200 | 80.13 ± 59.51 | 24.04 ± 17.85 |
| Nanodrop Onec | 80.58 ± 62.64 | 24.17 ± 18.79 |
| Qubit 1.0 | 30.88 ± 29.82 | 9.26 ± 8.95 |
| 2200 Tape Station | 44.85 ± 30.33 | 13.45 ± 9.1 |
| Qpcr | 32.6 ± 6.42 | 9.78 ± 1.93 |

**Table 1:** Average and standard deviation for concentration and yield values obtained in the 12 analysed samples with the Infinite F200, Nanodrop One C, Qubit 1.0 fluorometer, 2200 Tape Station and qPCR.

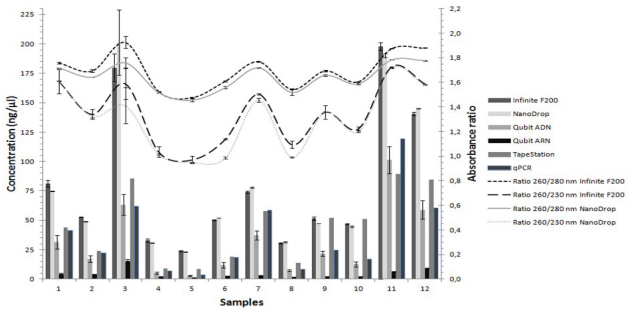


**Figure 4:** Concentration and purity results from 12 selected samples with the different tested methods. Error bars are indicated when multiple measurements were performed.

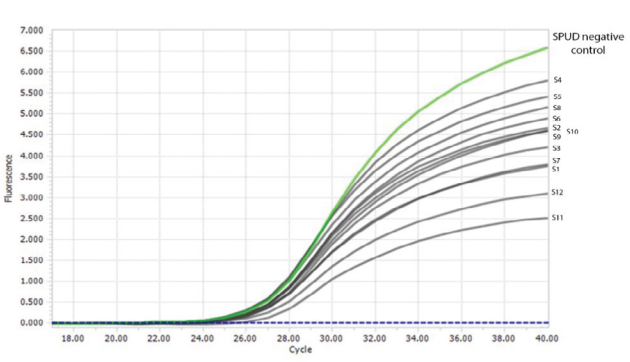


**Figure 5:** SPUD assay Cq results for 12 analyzed samples (S1-S12). SPUD negative control curve is indicated.

[3]. Otherwise, the pre-analytic management of specimens is a crucial step for downstream performance. It has been described that different extraction methods, as well as quantification and quality measurement methods used, can have an impact on the quality of the samples and the information derived from them, and therefore how they can be used later [8]. Commercial collection methods for saliva are usually used. In this work, an "in house" procedure has been implemented for DNA extraction from directly cryopreserved saliva by using a specific and commonly used kit for DNA extraction from blood. A high yield was obtained (52.58 ± 33.77 µg) according to previous studies performed with the saliva stabilization Oragene kit. So, a yield of 20.6 ± 3.52 µg was observed in male children and 17.1 ± 2.51 µg in females [20], while other authors described a yield of 10.8 µg (0.9 - 64.2 µg) [3] and 24 µg (0.2-52 µg) [15], or even, higher yields (154.9 ± 103.05) [21]. In the same sense, a high variability was obtained in our DNA yields, probably associated with the number of cells in saliva samples because of epithelial peeling, usually linked with the age and alterations and diseases of the oral mucosa [20]. When large-scale molecular studies are performed, it is essential to optimize not only collection and quantity but also quality of DNA in a time and cost-effective procedure for downstream applications [22]. In this work, purity values of 1.84 ± 0.12 and 1.56 ± 0.30 were observed for $A_{260}/A_{280}$ and $A_{260}/A_{230}$ ratios respectively. Similar results have been obtained in our lab with saliva samples collected with Oragene kit and processed with Chemagic MSMI robot using Chemagic DNA saliva kit Special (PerkinElmer, Inc) [6]. However, $A_{260}/A_{280}$ ratios of 1.63 (1.13 – 1.88) and 1.74 ± 0.07, and $A_{260}/A_{230}$ ratios of 0.80 (0.36–1.33) and 1.12 ± 0.2, were reported inspite of using Oragene kit [3,21]. A comparison of DNA qualification methods has also been presented in this manuscript in order to check the reliability and utility of spectrophotometric measures in the frame of a standard procedure. Coincident results were obtained for concentration and purity values with both the Infinite F200 and Nanodrop OneC spectrophotometric instruments. Also, similar concentration and yield results were obtained between Qubit 1.0 and qPCR, although considerably lower than for spectrophotometry. Finally, intermediate results were observed with the 2200 TapeStation. In relation to these results, contradictory conclusions have been achieved by different authors. Consistent measurements between Nanodrop and Qubit have been shown [23], while other authors concluded that spectrophotometry was the most precise method compared with fluorometry (Picogreen) and qPCR assays [5]. On the other hand and according to our results, additional works have shown that spectrophotometry overestimates DNA concentration [24,25] specifically when compared with densitometric analysis from agarose gel [8], and concordance between Qubit and qPCR results [8]. Fluorometry was much less influenced by contamination from proteins, salts, and RNA [5], while spectrophotometric measurements are affected by RNA traces [8,26]. On the contrary, spectrophotometry is not affected by DNA fragmentation [27], while fluorometry only detects dsDNA, but not ssDNA, degraded DNA or single nucleotides [28]. Because of this, similar spectrophotometric and fluorometric concentrations are obtained when high integrity DNA is present [6]. In this study, differences were not explained because of RNA contamination since a minimal presence of RNA in DNA samples was observed by Qubit 1.0. However, a certain degree of fragmentation in the DNA samples was revealed by the DIN values in spite of it having no remarkable impact on the amplification by real-time PCR of 3 different sizes gene targets (GAPDH, AF4 and ZFX). Fluorometry is more accurate based on our results as it is also supported by qPCR results which detect high molecular weight DNA in a selective way, due to no amplification of non-human DNA from contaminating sources, as for example, bacterial and viral DNA highly represented in saliva samples [5]. DNA template quality is one of the most important determinants for DNA amplification and assay reproducibility [14]. High failure rates of PCR analyses can be explained by the variability in DNA quantity and quality [20,29]. A high amplification success has been obtained previously by other authors with the Oragene saliva kit [30]. We performed the SPUD assay to check the absence of inhibitors after DNA isolation with the procedure proposed, with minimal PCR inhibition observed. Traditionally DNA integrity has not been objectively tested, and usually agarose gel has been used for integrity checking. In this manuscript, DNA integrity has been analysed by the 2200 Tape Station instrument in terms of DIN values and an innovative comparison between the DIN values and conventional DNA quality indicators is shown. It is highly recommended to perform a quality control of the input material, especially for expensive workflows such as NGS, as it saves time, effort and sample preparation overheads invested in low quality samples [22,31,32]. It has been established that samples with DIN >7 are acceptable to progress into the next step of library construction [17]. A DIN value of 6.84 ± 0.952 has been obtained in this study, and 35.7% of samples showed DIN > 7. Partial saliva DNA degradation has been observed previously in our lab using Oragene kit and processing samples with Chemagic MSMI robot using Chemagic DNA saliva kit Special (PerkinElmer, Inc) [6]. Collecting as many samples as possible is mandatory to establish DNA cohorts that represent the whole population and cover the genetic variants existing for different pathologies, in this way giving consistency to the studies carried out. The development of a non-invasive methodology with a moderate cost is a very useful tool for this purpose.

## Conclusion

The procedure described in this manuscript guarantees a high amount of DNA from saliva samples valid for any downstream molecular application, with an important reduction in costs. In this sense, the rough cost per extraction is set at 12 euros, while the costs are 29.5 euros with the Oragene kit. The integrity analysis (DIN value) supposes additional information relevant to the conventional DNA quality indicators. The main drawback of our procedure is that the saliva samples must be immediately frozen once collected until processing, while the Oragene kit allows keeping the sample at room temperature for up to 5 years.

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